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In addition to fostering cancer cell invasion, matrix metalloproteinases can affect cellular signaling, and may contribute to early tumor development. Functionally normal cultured mammary epithelial cells transfected with a tetracycline (Tet)-repressible stromelysin-1 (Str1) cDNA undergo epithelial-to-mesenchymal conversion following induction of Str1 expression or treatment with recombinant enzyme. cDNA array profiling also indicates that Str1 causes coordinated changes in the expression of intermediate filament markers and regulators of cancer development. When the cells are injected into surgically cleared mammary fat pads of immunodeficient mice, they form epithelial glandular structures when Str1 expression is repressed by adding Tet to the drinking water, but form infiltrative mesenchymal-like tumors when Str1 is induced by withholding Tet. Once initiated, the tumors become independent of continued Str1 expression. Using a second genetic approach, transgenic mice that express Str1 in mammary epithelium develop fibrosis (77%), hyperplasias (64%), dysplasias (20%) and carcinomas (7%), whereas non-transgenic controls develop only mild fibrosis and/or hyperplasia (<12%, p<0.0001).

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Appended Manuscripts:

Introduction

Matrix metalloproteinases (MMPs) are invariably upregulated in the stromal compartment of epithelial cancers and appear to promote invasion and metastasis. Here we report that phenotypically normal mammary epithelial cells with tetracycline-regulated expression of MMP-3/stromelysin-1 (Str1) form epithelial glandular structures *in vivo* without Str1, but form invasive mesenchymal-like tumors with Str1. Once initiated, the tumors become independent of continued Str1 expression. Str1 also promotes spontaneous premalignant changes and malignant conversion in mammary glands of transgenic mice. These changes are blocked by co-expression of a *TIMP1* transgene. The premalignant and malignant lesions have stereotyped genomic changes unlike those seen in other murine mammary cancer models. These data indicate that Str1 influences tumor initiation and alters neoplastic risk.

Body

We have examined how Str1 affects tumor progression using two genetic approaches: phenotypically normal mammary epithelial cells that express Str1 in a tetracycline-regulated manner, and an Str1 transgene targeted to mouse mammary glands by the mouse whey acidic protein (WAP) gene promoter. In the tetracycline-regulated cells, Str1 triggered the phenotypic conversion of functionally normal mammary epithelial cells into invasive, tumorigenic, mesenchymal-like cells both in culture and *in vivo*. Str1 was also able to modulate the expression of genes, such as cyclin D1, that are either known or thought to promote tumor development. More remarkably, we observed the development of spontaneous premalignant lesions and mammary cancers in the WAP-Str1 transgenic mice and the virtual absence of such changes in their nontransgenic littermates and in related bitransgenic mice that co-express a human tissue inhibitor of metalloproteinases (TIMP-1) transgene under the control of the same promoter. These Str1-induced changes, which occur in the absence of exogenous mutagens or endogenous oncogene or suppressor gene defects, offer strong evidence that Str1 can act as a natural tumor promoter. These data are described in a recently published research article (Sternlicht *et al.*, 1999) and in a review in press; each of which are appended to this report.

Briefly, treatment of an immortal, but functionally normal, mouse mammary epithelial cell line (Scp2) with recombinant Str1 induced an epithelial-to-mesenchymal phenotypic transition, characterized by loss of cell-cell interactions, acquisition of a scattered morphology, downregulation of epithelial cytokeratins, and upregulation of the mesenchymal marker vimentin. These changes did not occur when a synthetic MMP inhibitor was also added. Similar changes have been observed after the induction of Str1 expression by tetracyclin withdrawal in tetracyclin-regulated Scp2 clones (Lochter *et al.* (1997) J. Cell Biol. 139:1861-72). When these cells were

injected into surgically cleared (gland-free) mammary fat pads of immunocompromised mice and Str1 expression was blocked by adding tetracyclin to the drinking water, the injected cells formed gland-like cysts and duct-like structures that were patent and cytokeratin-positive, but vimentin-negative. However, when Str1 expression was induced by tetracyclin withdrawal, the injected cells formed highly infiltrative spindle-cell tumors that were mostly cytokeratin-negative and vimentin-positive. Thus, epithelial-to-mesenchymal conversion and the acquisition of an invasive and tumorigenic phenotype had occurred *in vivo* in the presence of Str1, but not in its absence.

In WAP-Str1 transgenic mice, we previously observed precocious lobulo-alveolar development in 3-10 week-old animals (Sympson *et al.* (1994) J. Cell Biol. 125:681-93) and a reactive stroma in 2-4 month-old mice (Thomasset *et al.* (1998) Am. J. Pathol. 153:547-67). Here we found that 77% of 6-24 month-old mice (median age, 18 months) had moderate or severe mammary fibrosis, 64% had moderate or severe mammary hyperplasia, 53% had lymphocytic infiltrates, 20% had atypical hyperplasias and 7.4% developed mammary carcinomas with an average tumor latency of 18.7 months. On the other hand, nontransgenic littermate controls (median age, 18 months) exhibited a low incidence of mild hyperplasia, fibrosis and lymphocytic infiltration, and none of the more severe lesions. WAP-Str1 mice were also mated with mice carrying a TIMP1 transgene under the control of the same promoter, and mammary hyperplasias were examined as a surrogate end-point in 10-16-month-old female offspring. Only 19% of mice carrying both Str1 and TIMP1 transgenes had mild mammary hyperplasias, whereas 73% of mice carrying the Str1 transgene alone had moderate-to-severe hyperplasias, indicating that active Str1 is required for mammary lesions to develop. Analysis of ten mammary lesions from three WAP-Str1 transgenic lines by comparative genomic hybridization revealed DNA losses in specific regions of mouse chromosomes 4 and 7 in both premalignant and malignant lesions, and DNA copy number gains on chromosomes 6 and 15 in a severe hyperplasia and three tumors that had undergone epithelial-to-mesenchymal conversion. Despite using a fairly small sample size and a statistic (Fisher's exact test) that ignores consistency in direction and sub-chromosomal localization, the genomic changes on chromosomes 4, 7, 6 and 15 were remarkably nonrandom ($p<0.001$, 0.01, 0.05 and 0.05, respectively). DNA gains on chromosome 15 were also seen in the tetracyclin-regulated cells that had undergone epithelial-to-mesenchymal conversion.

Additional, failed, unfinished or pending experiments

Effect of Str1 on other cell lines. Previously, we examined the effect of recombinant Str1 on other mouse mammary epithelial cells lines with little success (see September 1998 report). We have now established other mammary epithelial cell lines that express Str1 in a tetracycline-regulated fashion. To date, however, they have not shown altered behavior in culture or *in vivo*.

Effect of other MMPs on mammary epithelial cell lines. We have also developed autoactivating forms of MMP9/gelatinase B and MMP13/collagenase-3 and have derived mammary epithelial cell lines (from the mouse NMuMG and human MCF-7 lines) that express these enzymes in a tetracycline-regulated manner. Thus far, neither enzyme appears to promote appreciable phenotypic changes in these cells in culture, although MMP13-expressing MCF-7 clones have shown a subtle tendency to form larger tumors with a more infiltrative border *in vivo.*

Effect of genetic complementation on neoplastic progression. Although coexpression of a TIMP1 transgene quenches neoplastic progression in the WAP-Str1 transgenic mice, we have not undertaken further genetic crosses with MMP knockout mice due to the prolonged tumor latency and low tumor incidence in these mice. Instead, we have entered into a collaboration with Dr. Craig McArthur to investigate the effect of various MMPs on mammary tumorigenesis in MMTV-FGF8b transgenic mice. We are also considering other mouse models of mammary cancer.

Effect of Str1 on E-cadherin/β-catenin signaling. Because Str1 triggers the cleavage of E-cadherin, the cytoplasmic redistribution of β-catenin, and the upreglation of cyclin D1, a β-catenin-regulated oncogene, we have undertaken experiments to investigate the role of Str1 in E-cadherin/β-catenin/Lef signaling. Initial experiment using a fos promoter-driven luciferase reporter construct and an alcohol dehydrogenase promoter-driven CAT reporter construct containing upstream wild-type or mutant Lef regognition sequences were unsuccessfull. We are now undertaking similar experiments using cyclin D1 promoter-driven reporter constructs.

Key Research Accomplishments

- Str1 can promote spontaneous premalignant changes and mammary carcinogenesis in transgenic mice.
- Tissue inhibitor of metalloproteinases-1 (TIMP-1) inhibits mammary neoplasia in WAP-Str1 transgenic mice.
- Str1 can convert functionally normal mammary epithelial cells into invasive, tumorigenic, mesenchymal-like cells in culture and *in vivo.*
- Str1 can modulate the expressions of genes that control tumor development.
- Str1 can promote the acquisition of nonrandom genomic changes.

Reportable Outcomes

Journal Articles

Sternlicht MD, Lochter A, Sympson CJ, Huey B, Rougier JP, Gray JW, Pinkel D, Bissell MJ and Werb Z. (1999) The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell*, **98**, 137-146.

Reviews

Lochter A, **Sternlicht MD**, Werb Z and Bissell MJ. (1998) The significance of matrix metalloproteinases during early stages of tumor progression. *Ann. N. Y. Acad. Sci.*, **857**, 101-118.

Sternlicht MD, Bissell MJ and Werb Z. (1999) The matrix metalloproteinase stromelysin-1 acts as a natural mammary tumor promoter. *Oncogene*, in press.

Book Chapters

Sternlicht MD and Werb Z. (1999) Extracellular Matrix Proteinases. In: *Guidebook to the Extracellular Matrix, Anchor and Adhesion Proteins.* T Kreis and R Vale (eds). Oxford University Press, New York. pp.503-603.

Sternlicht MD, Coussens LM, Vu TH and Werb Z. (1999-2000) Biology and Regulation of the Matrix Metalloproteinases. In: *Matrix Metalloproteinase Inhibitors in Cancer Therapy.* N.J. Clendeninn and K. Appelt, editors. Humana Press, Totowa, NJ. in press.

Abstracts (for platform presentations, the presenting author is underlined)

Sternlicht MD, Lukashev M, Lochter A, Bissell MJ and Werb Z. (1998) Regulation of early and late mammary tumorigenesis by the matrix metalloproteinase stromelysin-1. *Proc. Sixth SPORE Investigators' Workshop,* Rockville, MD, #50.

Werb Z, Coussens L, Lukashev M, **Sternlicht MD**, and Vu T. (1998) Genetic dissection of protease function in tumor progression. *Proc. The Thirteenth Aspen Cancer Conference Workshop: Mechanisms of Toxicity and Carcinogenesis,* Aspen, CO, #3.

Bissell MJ, Simian M, **Sternlicht MD**, Werb Z and Lochter A. (1998) Central role of extracellular matrix and extracellular matrix-degrading enzymes in mammary gland branching morphogenesis, epithelial-to-mesenchymal conversion and cancer. *Proc. Proteases and Protease Inhibitors in Cancer, an AACR/APMIS meeting,* Nyborg, Denmark, #20.

Sternlicht MD, Lukashev M, Lochter A, Bissell MJ and Werb Z. (1998) MMP-3/stromelysin-1 promotes mammary carcinogenesis and cancer progression. *Proc. Mechanisms of Tumor Growth and Invasion Mediated by Proteolysis, a UCSF Molecular Design Institute Conference,* San Francisco.

Sternlicht MD, Lukashev M, Lochter A, Bissell MJ and Werb Z. (1998) MMP-3/stromelysin-1 promotes early and late tumorigenesis. *Mol. Biol. Cell,* **9(S)**, 6a(#32).

Sternlicht MD, Lukashev M, Lochter A, Bissell MJ and Werb Z. (1999) The stromal enzyme MMP-3/stromelysin-1 promotes mammary carcinogenesis. *Proc. AACR Special Conference on the Mutant Mouse in Cancer,* Keystone, CO, #C36.

Sternlicht MD, Bissell MJ and Werb Z. (1999). The WAP-stromelysin-1 transgenic mouse. *NCI Workshop on Comparative Pathology of Animal Models for Mammary Cancer,* Annapolis, MD, March 3-5, 1999.

Sternlicht MD, Lukashev M, Lochter A, Bissell MJ and Werb Z. (1999). Stromelysin-1 promotes mammary carcinogenesis. *1999 Gordon Research Conference on Matrix Metalloproteinases*, Colby-Sawyer College, New London, NH, August 8-13, 1999.

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The Stromal Proteinase MMP3/Stromelysin-1 Promotes Mammary Carcinogenesis

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Summary

Matrix metalloproteinases (MMPs) are invariably upregulated in the stromal compartment of epithelial cancers and appear to promote invasion and metastasis. Here we report that phenotypically normal mammary epithelial cells with tetracycline-regulated expression of *MMP3/stromelysin-1* (*Str1*) form epithelial glandular structures in vivo without *Str1* but form invasive mesenchymal-like tumors with *Str1*. Once initiated, the tumors become independent of continued *Str1* expression. *Str1* also promotes spontaneous premalignant changes and malignant conversion in mammary glands of transgenic mice. These changes are blocked by coexpression of a *TIMP1* transgene. The premalignant and malignant lesions have stereotyped genomic changes unlike those seen in other murine mammary cancer models. These data indicate that *Str1* influences tumor initiation and alters neoplastic risk.

Introduction

Extracellular matrix (ECM)-degrading matrix metalloproteinases (MMPs) are universal features of carcinoma progression and are associated with tumor angiogenesis, invasion, and metastasis. MMPs not only foster invasion and spread by disrupting ECM barriers but also affect cellular signaling by several routes (Werb, 1997; Lukashev and Werb, 1998). Interestingly, most MMPs are synthesized not by the genetically altered cancer cells but by adjacent and intervening stromal cells (Coussens and Werb, 1996). There is also a growing awareness that stromal cells and the matrix microenvironment can influence initial tumor development (Jacoby et al., 1997; Howe et al., 1998; Hsieh et al., 1998; Jacobs et al., 1999). Thus, given their stromal origin, consistent upregulation, and signaling capacity, stromal MMPs may also contribute to the initial stages of cancer development.

MMP3/stromelysin-1 (Str1) is a candidate for a stromal MMP that can exert oncogenic effects. It can degrade numerous ECM substrates, including collagens III, IV, V, IX, X, and XI, laminins, elastin, entactin, fibronectin, fibrin, fibrillins, fibulin, link protein, osteonectin, tenascin, vitronectin, and ECM proteoglycans (reviewed in Sternlicht and Werb, 1999). Str1 can also release cell surface molecules, including E-cadherin, L-selectin, heparin-binding EGF-like growth factor, and TNF-α; it can activate other MMPs, including gelatinase B and the collagenases; and it can inactivate several serine proteinase inhibitors (reviewed in Sternlicht and Werb, 1999). Importantly, *Str1* was originally cloned (Matrisian et al., 1985) and repeatedly recloned as a tumor-specific gene (Muller et al., 1988; Ostrowski et al., 1988).

Str1 is expressed in stromal cells throughout mammary development and is maximally expressed during involution when ECM remodeling and alveolar regression take place (Talhouk et al., 1992; Witty et al., 1995; Lund et al., 1996). Although stromal cells synthesize Str1 in vivo, the protein associates with the epithelium (Talhouk et al., 1992; Lund et al., 1996). There are two distinct responses to Str1 in mammary epithelium: proliferation and branching in ductal cells, and apoptosis in differentiated secretory alveolar cells (Sympson et al., 1994; Boudreau et al., 1995; Witty et al., 1995). There are also parallels between development and neoplasia. Expression of *Str1* in the mammary epithelium of transgenic mice during development induces upregulation of endogenous *Str1* and other MMPs in surrounding stromal fibroblasts and leads to fibrosis, neovascularization, and tenascin-C expression, all of which are hallmarks of the reactive stroma of involution (Alexander et al., 1996; Thomasset et al., 1998). Increased cell proliferation, an altered stroma, angiogenesis, and tenascin-C expression are also features of cancer progression (Borsi et al., 1992; Rønnov-Jessen et al., 1996).

Here, we have examined how *Str1* affects tumor progression using two genetic approaches: phenotypically normal mammary epithelial cells that express *Str1* in a tetracycline-regulated manner, and an *Str1* transgene targeted to mouse mammary glands by the mouse whey acidic protein (WAP) gene promoter. Our results indicate that not only can *Str1* induce an altered stromal environment, but as a product of such an environment, it can promote the phenotypic conversion and malignant transformation of mammary epithelial cells.

Results

Str1 Promotes Epithelial-to-Mesenchymal Transitions in Culture

Str1 has both ECM and cell surface targets (Sternlicht and Werb, 1999). Thus, its biologic effects could arise from its alteration of surrounding ECM or by its direct action on the epithelial cells themselves. Therefore, we treated an immortal but functionally normal mouse mammary epithelial cell line (Scp2), which responds to ECM signals but does not assemble its own ECM, with recombinant Str1 (rStr1). Scp2 cells grow as an epithelial

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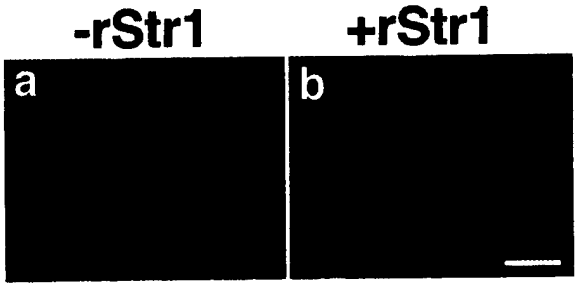

Figure 1. Effect of Str1 on Morphology and Intermediate Filament Expression in Scp2 Cells

Cells were maintained for 6 days in the (a) absence or (b) presence of activated recombinant Str1 (rStr1) and stained by indirect immunofluorescence for cytokeratins (red) and vimentin (green). Nuclei were counterstained with DAPI (blue). Scale bar, 50 μm.

sheet with E-cadherin-rich adherens junctions and are cytokeratin positive and vimentin negative. The rStr1 rapidly induced epithelial-to-mesenchymal transitions (EMT), characterized by loss of cell–cell interactions, acquisition of a scattered morphology, downregulation of epithelial cytokeratins, and upregulation of the mesenchymal marker vimentin (Figure 1). These changes did not occur when the synthetic MMP inhibitor GM6001 was also added (data not shown). This treatment of the cells in *trans* mimics the in vivo production of Str1 by fibroblasts acting on mammary epithelium.

Str1 Promotes EMT and Tumorigenicity In Vivo

To examine the effect of Str1 on Scp2 cells in vivo, we used Scp2 cells stably transfected with an autoactivating rat *Str1* cDNA under the control of a tetracycline (Tet)-repressible promoter (Lochter et al., 1997). These transfected cells grow as epithelial sheets when grown in medium containing Tet, whereas induction of *Str1* expression by Tet withdrawal results in EMT, cleavage and loss of E-cadherin, and acquisition of the ability to form anchorage-independent colonies in agar and invade Matrigel (Lochter et al., 1997). For the present study, we used two clones with regulated *Str1* expression (p2S7 and p2S10), a nonexpressing clone (p2S3), and parental Scp2 cells. When Scp2 cells and *Str1*-transfected cells grown in the presence of Tet were injected into surgically cleared (gland-free) mammary fat pads of *scid/scid* mice, they grew and formed relatively normal duct-like and pseudoglandular structures if the mice were given Tet in their drinking water (Figures $2A_a$ and $2A_b$). These structures had cytokeratin-8-positive, vimentin-negative luminal cells but lacked smooth muscle actin-positive myoepithelial cells that normally surround luminal epithelium (Figures $2A_c$ and $2A_d$). The ductal structures did not branch, which is consistent with the lack of myoepithelial cells that produce epimorphin, a morphogen required for branching (Hirai et al., 1998). This growth pattern persisted for at least 13 weeks. However, when *Str1* expression was induced in vivo by withholding Tet from the drinking water, the p2S10 cells formed small tumors in 5 of 18 injected sites by 6 weeks (Figure 2B). The tumors were composed largely of vimentin-positive and cytokeratin-negative spindle-shaped (mesenchymal-like) cells and were invasive at their periphery (Figure 2A). Thus, induction of *Str1* expression triggered EMT and rendered the cells tumorigenic and invasive in vivo.

When Tet was withheld from the drinking water for 12 days after injecting uninduced p2S10 cells and then replaced for the remaining 4.3 weeks, tumor formation was still seen, albeit at a lower rate than when Tet was continuously absent (Figure 2B). This suggests that once tumorigenicity is achieved, it would not be blocked by repressing *Str1* expression. To test this, we induced *Str1* expression in culture for 2 months in p2S10 and p2S7 cells. When the preinduced cells were injected into cleared fat pads, large tumors grew at all injected sites regardless of the presence or absence of Tet in the drinking water (Figure 2B). The tumors were highly invasive and composed almost entirely of vimentin-positive spindle-shaped cells with fewer than 1% cytokeratin-positive cells (Figure $2A_{e-h}$). Interestingly, several tumors had small areas of differentiation to a cartilage-like phenotype (Figure $2A_{e-h}$). Such changes, called chondroid metaplasia, are also seen in human tumors with EMT and were first described three centuries ago (cited in Wargotz and Norris, 1989). Thus, once EMT is induced, converted cells no longer require *Str1* transgene stimulation to elicit an altered program of gene expression and tumorigenesis.

The preinduced p2S10 cells also acquired the ability to form subcutaneous tumors with or without Tet in the drinking water (data not shown). These tumors were about one-tenth the size of the orthotopic tumors but had the same infiltrative spindle-cell morphology. No other cells grew subcutaneously. Taken together, these data indicate that Str1 can trigger tumor progression in an immortal but functional mammary epithelial cell line.

Str1 Promotes the Development of Premalignant and Malignant Mammary Lesions in Transgenic Mice

To examine the long-term effect of Str1 on normal mammary epithelial cells, we used WAP-*Str1* transgenic mice. We had previously observed precocious lobuloalveolar development in 3- to 10-week-old WAP-*Str1* transgenic mice (Sympson et al., 1994) and an altered reactive stroma in 2- to 4-month-old mice (Thomasset et al., 1998). Here we found that 6- to 24-month-old transgenic mice exhibited mammary abnormalities, premalignant lesions, and malignancies (Figures 3 and 4). Of 163 mice from five independent transgenic lines (median age 18 months), 77% had moderate or severe fibrosis (collagen and fibroblast accumulation with adipocyte loss), 64% had moderate or severe hyperplasia (epithelial cell accumulation), 53% had lymphocytic infiltrates, 20% had dysplasias (atypical proliferative lesions) or ductal carcinoma in situ, and 7.4% developed mammary carcinomas.

The incidence of the various lesions was 1.2- to 1.9-fold higher in parous mice than in virgin mice, and hyperplastic and fibrotic changes were generally more severe in parous animals. Whereas increases in incidence and severity would be expected given the pregnancy-driven nature of the WAP promoter, their modesty may reflect the fact that the promoter is minimally active during each estrus cycle, so that parity only slightly increases

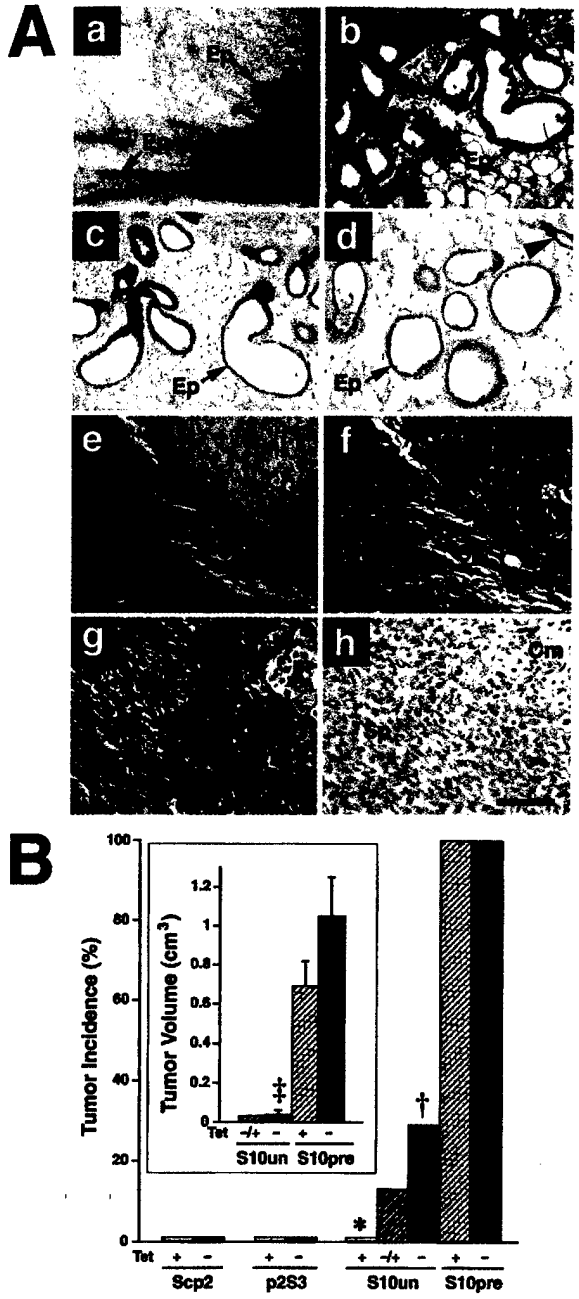

Figure 2. Effect of Str1 on Mammary Epithelial Growth In Vivo

(A) Histologic appearance of p2S10 mammary epithelial cells grown in cleared mammary fat pads. (a–d) Appearance of epithelial ductal and gland-like structures (Ep) that form in the absence of *Str1* expression as seen by (a) whole-mount, (b) H&E, (c) anti-cytokeratin-8, and (d) anti-smooth muscle actin staining. The arrowhead in (d) indicates vascular smooth muscle cells. (e–h) Appearance of spindle-cell tumors (Sp) that form when *Str1* expression is induced in vivo or prior to injection as seen by (e) H&E, (f) Alcian blue, (g) anti-vimentin, and (h) anti-cytokeratin-8 staining. Areas of cartilage (chondroid metaplasia, Cm) are present in the upper right corner (e–h). Scale bars, (a) 200 μm and (b–h) 100 μm.

(B) Tumor incidence (percent of injected sites) and volume (cm^3; mean ± SEM) following injection of parental (Scp2), nonexpressing (p2S3), uninduced (S10un), and preinduced (S10pre) cells into cleared mammary fat pads in mice maintained for 6 weeks with (+) or without (−) Tet in their drinking water or without Tet for the first 12 days only (−/+). *, $p < 0.0001$ versus S10pre cells in mice maintained with or without Tet (two-tailed Fisher's exact test); †, $p < 0.05$ versus S10un cells in mice maintained with Tet and $p < 0.001$ versus S10pre cells in mice maintained with or without Tet (Fisher's exact test); ‡, $p < 0.005$ and $p < 0.001$ versus S10pre cells in mice maintained with and without Tet, respectively (t test).

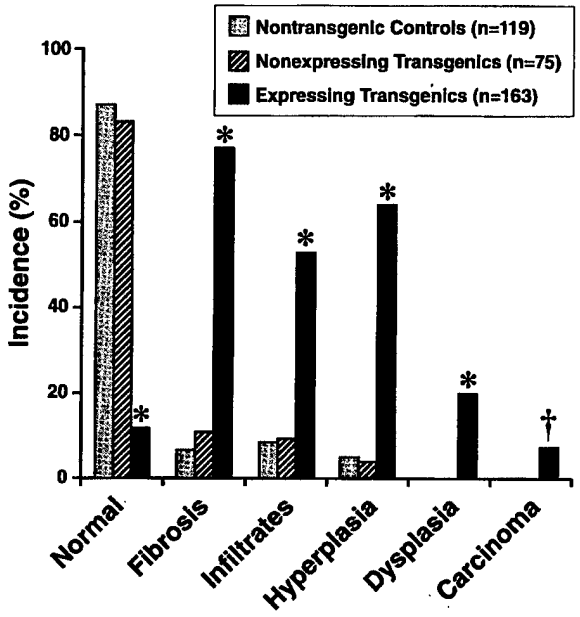

Figure 3. Incidence of Mammary Gland Pathologies in *Str1* Transgenic Mice

The *Str1*-expressing transgenics include 100, 31, 16, 9, and 7 mice from five independent transgenic lines, respectively. *, $p < 0.00001$ versus nontransgenic or nonexpressing transgenic controls (two-tailed Fisher's exact test); †, $p = 0.002$ and $p = 0.02$ versus nontransgenic and nonexpressing transgenic controls, respectively (Fisher's exact test).

lifetime Str1 exposure. Indeed, even low transgene expression during puberty causes increased ductal branching and precocious lobuloalveolar development (Sympson et al., 1994), thus increasing the number of potential target cells that are "at risk" over an extended period of time. Average tumor latency was 18.7 months, with the first tumor appearing in a 6-month-old parous mouse. The hyperplastic and fibrotic lesions tended to be more severe in older animals, and the incidence of hyperplasia increased from 46% at 6 months to 78% at 2 years of age. These data are consistent with a model of multistage neoplastic progression induced by *Str1* expression.

Mammary carcinomas were seen in mice from three different founders (fewer than ten mice were studied in the other two lines). Nine of the tumors were moderately well-differentiated adenocarcinomas (Figure 5_{a-c}), and three were undifferentiated tumors with evidence of EMT. Two of these were carcinosarcomas with cytokeratin-positive and vimentin-negative epithelial-like populations and distinct vimentin-positive and cytokeratin-negative fibroblast-like populations (Figure 5_{g-i}). The third undifferentiated tumor gave rise to lung and kidney metastases and expressed both cytokeratins and vimentin (Figure 5_{d-f}). A cell line derived from the primary

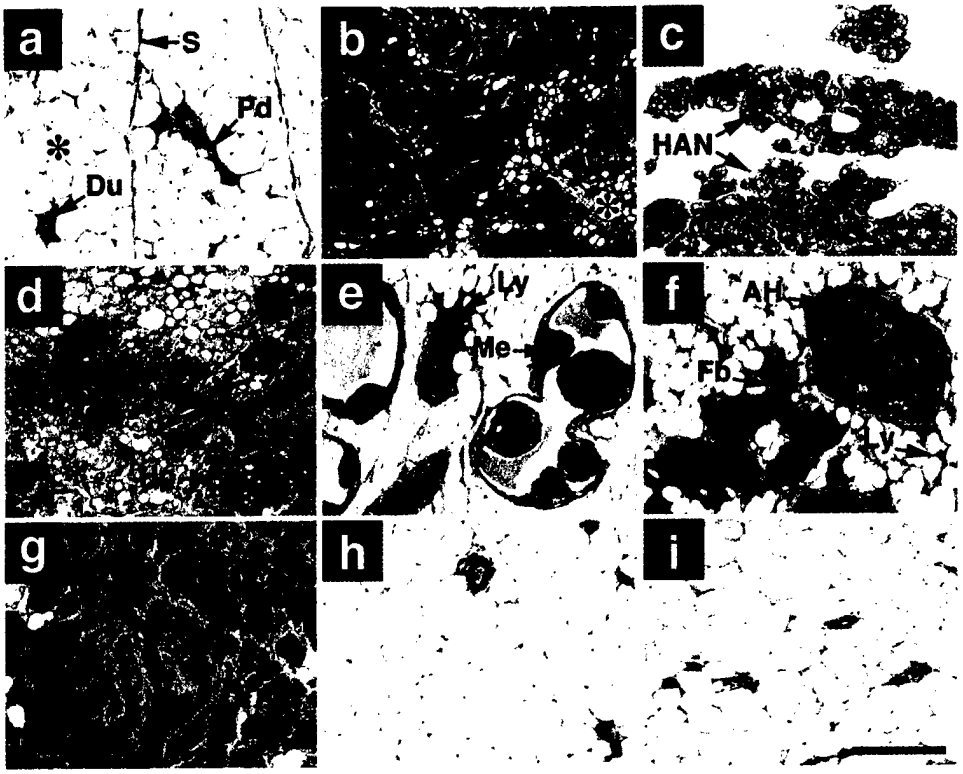

Figure 4. Histologic Appearance of Normal and WAP-*Str1* Mammary Glands

Histologic sections are from (a) nontransgenic, (b–g) WAP-*Str1* transgene–expressing, (h) WAP-*Str1* transgene–nonexpressing, and (i) *Str1/TIMP1* double transgene–positive female mice.

(a) Normal mammary gland with resting ducts (Du), abundant adipose tissue (asterisk), and minimal periductal (Pd) and septal (S) collagen (stained blue).

(b) Severe hyperplasia (Hp) with considerable intervening fibrosis (Fb; stained blue) and multilocular adipocytes (asterisk).

(c) Hyperplastic alveolar nodule (HAN) with lipid droplets characteristic of secretory activity even though this gland comes from a nulliparous mouse.

(d) Multifocal alveolar hyperplasia (Hp) with eosinophilic (pink) fibrotic areas and multilocular adipocytes (asterisk).

(e) Intraductal papillary hyperplasia with lymphocytic infiltrates (Ly). The small hyperchromatic cells (Me) were cytokeratin-8 negative and smooth muscle actin positive, indicating the abnormal presence of myoepithelial cells within the severely distended ducts.

(f) Atypical hyperplasia (AH) with lymphocytic infiltrates (Ly) and mild fibrosis (Fb).

(g) Atypical hyperplasia with considerable fibrosis.

(h and i) Normal mammary histology seen with the loss of *Str1* transgene expression or its inhibition by TIMP1, respectively.

(a and b) Masson's trichrome. (c–i) Hematoxylin/eosin. Scale bar, 200 μm.

mammary tumor also coexpressed cytokeratin and vimentin and formed invasive tumors that were cytokeratin and vimentin positive in vivo (data not shown).

In contrast to the *Str1*-expressing transgenic mice, nontransgenic littermate controls (median age 18 months) exhibited a low incidence of mild hyperplasia, fibrosis, and lymphocytic infiltration (Figure 3). These low incidence rates were similar for virgin and parous controls. We also isolated two sublines of *Str1* transgenic mice in which expression of the transgene was silenced and could not be detected by RT-PCR, presumably due to transgene methylation as determined by altered sensitivity to restriction endonucleases. The nonexpressing transgenic mice (median age 13 months) also showed a low incidence of mild lesions (Figure 3). These data indicate that expression of the *Str1* transgene is required to promote neoplastic progression.

TIMP1 Inhibits the Development of Mammary Hyperplasias in *Str1* Transgenic Mice

If the induction of neoplasia by Str1 is due to its proteolytic activity, then overexpression of its endogenous inhibitor, tissue inhibitor of metalloproteinases-1 (TIMP1), should quench this phenotype. Thus, we mated WAP-*Str1* mice with mice carrying a human *TIMP1* transgene under the control of the same WAP promoter to examine the effect of TIMP1 on the development of mammary lesions in WAP-*Str1* mice. Mammary hyperplasias were examined as a surrogate endpoint in 10- to 16-month-old female offspring (Figure 6). Only 3 of 16 mice (19%) carrying both *Str1* and *TIMP1* transgenes had mild mammary hyperplasias, whereas 8 of 11 mice (73%) carrying the *Str1* transgene alone had moderate to severe hyperplasias. A similar frequency (66%) was seen in the cohort of 100 WAP-*Str1* females from the same transgenic founder (M2-5). Mice carrying only the *TIMP1* transgene had a slightly altered involution phenotype but were otherwise normal. Nontransgenic littermates had entirely normal mammary glands. These data indicate that active Str1 is required for mammary lesions to develop.

Str1 Expression Promotes Stereotyped Genomic Changes

Because Str1 acts extracellularly, we wanted to determine if tumorigenicity was accompanied by genomic

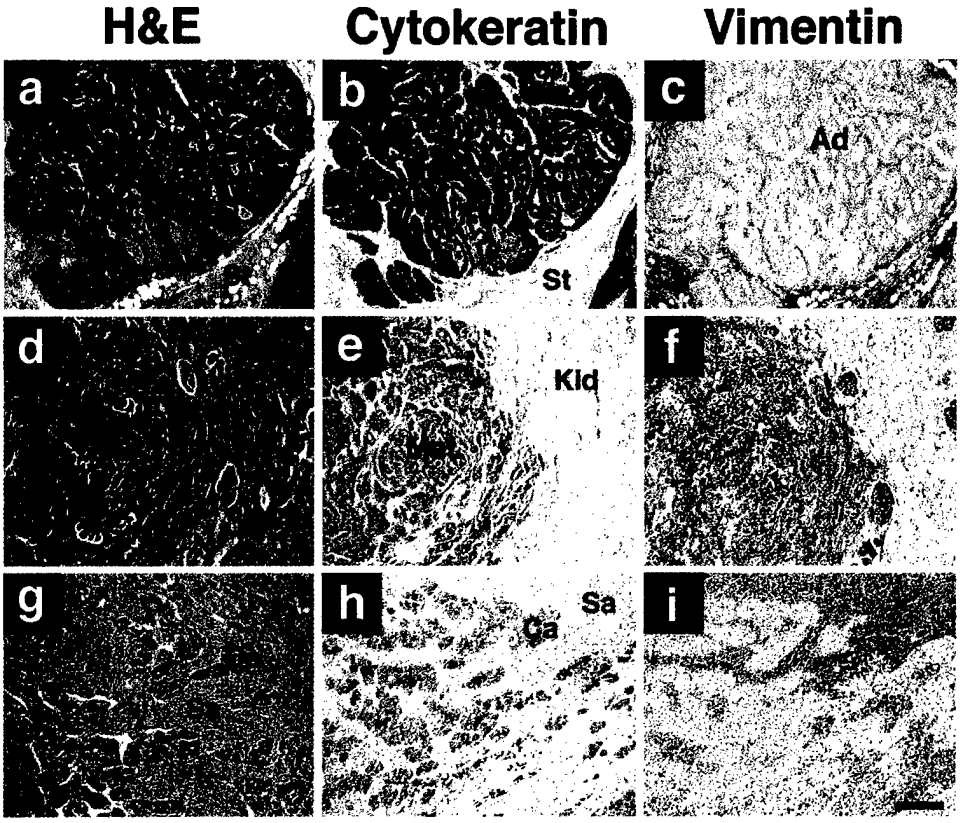

Figure 5. Histologic Appearance of Malignant Mammary Tumors from *Str1* Transgenic Mice

(a–c) Moderately differentiated adenocarcinoma (Ad) with adjacent and intervening vimentin-positive stromal cells (St).
(d–f) Renal metastasis (Met) from an undifferentiated mammary carcinoma. Normal kidney (Kid) is present on the right.
(g–i) Carcinosarcoma with distinct carcinomatous (Ca) and sarcomatous (Sa) areas exhibiting epithelial and mesenchymal features, respectively. H&E, hematoxylin/eosin stains. Scale bar, 200 μm.

reorganization. Analysis of ten mammary lesions from three *Str1* transgenic lines by comparative genomic hybridization (CGH) revealed DNA losses in specific regions of mouse chromosomes 4 and 7 in both premalignant and malignant lesions (Figure 7A). In addition, the three tumors with EMT and a severe hyperplasia had DNA copy number gains on chromosomes 6 and 15. Because these gains were associated with EMT, we separately analyzed the epithelial- and mesenchymal-like populations of one carcinosarcoma after laser microdissection. The chromosome 15 amplification was only seen in microdissected fibroblast-like areas that had undergone EMT, whereas other CGH changes were seen throughout the tumor. Because the DNA from the severe hyperplasia with gains on chromosomes 6 and 15 came from an area of tissue that was not expressly visualized and microdissected, its genomic changes may reflect those of an occult tumor with EMT. CGH profiles for nonneoplastic tissues from the same ten mice (data not shown) and from two histologically normal mammary glands from separate transgenic lines were invariably normal. Thus, even with this sample size and despite using a statistic (Fisher's exact test) that ignores consistency in direction and subchromosomal localization, the genomic changes on chromosomes 4, 7, 6, and 15 were remarkably nonrandom ($p < 0.001$, 0.01, 0.05, and 0.05, respectively).

Independent evidence for an association of mouse chromosome 15 with EMT was obtained from the p2S cells and their tumors. DNA gains in chromosome 15 were seen in the preinduced p2S10 and p2S7 cells and their tumors but not in the parental cells (Figure 7B). Identical gains in the middistal portion of chromosome 15 were seen in both microdissected spindle-cell and cartilage-like areas of p2S10 tumors but not in adjacent normal stroma, indicating that both areas arose from injected rather than host cells and that the cartilage-like areas represent a further manifestation of EMT. The preinduced cells and their tumors also showed amplifications on chromosomes 3, 5, and 11. Unlike the WAP-*Str1* mammary tumors, no changes were seen on chromosomes 4, 7, or 6. Thus, although we studied relatively few samples, our data further implicate mouse chromosome 15 as significant for Str1-induced tumors that have undergone EMT.

Discussion

Stromal MMPs Alter Neoplastic Risk

An altered stromal environment appears to presage cancer development. In the case of WAP-*Str1* transgenic mice, stromal defects appear (Thomasset et al., 1998) well before neoplastic changes are observed. In humans, cancer susceptibility is increased in certain fibrotic and chronic inflammatory conditions (Hsieh et al., 1998; Jacobs et al., 1999). Moreover, some inherited

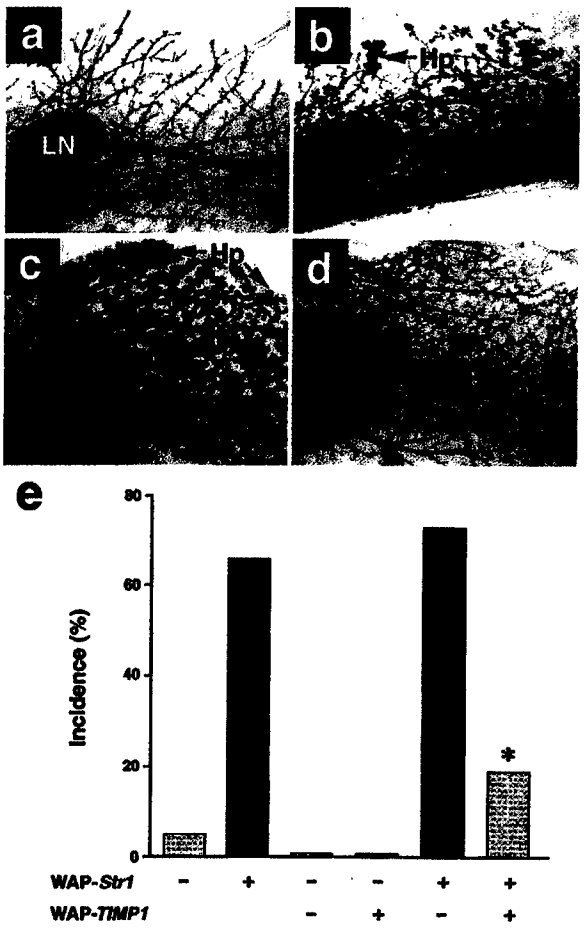

Figure 6. Effect of a *TIMP1* Transgene on *Str1* Transgene–Induced Mammary Hyperplasias as Seen by Whole-Mount Staining

(a) 16-month-old nontransgenic control.

(b) 16-month-old WAP-*Str1* transgenic mouse with multifocal alveolar hyperplasia (Hp).

(c) 12-month-old WAP-*Str1*-positive/WAP-*TIMP1*-negative mouse with diffuse alveolar hyperplasia.

(d) 12-month-old *Str1/TIMP1* double transgenic mouse from the same litter as the mouse in (c). The mammary gland in (d) was judged to be within normal limits by whole-mount and hematoxylin/eosin staining. The glands shown are from mice that had undergone a single pregnancy and lactation at least 5 months prior to sacrifice. LN, lymph node. Scale bar, 500 μm.

(e) Incidence of mammary hyperplasias in double and single transgenic mice. The single transgenic mice are from the related M2-5 line. Gray and black bars indicate mild and moderate to severe hyperplasias, respectively. *, $p < 0.02$ versus littermates carrying the *Str1* transgene alone and $p < 0.0006$ versus the large cohort of M2-5 *Str1* transgenics, respectively (two-tailed Fisher's exact test).

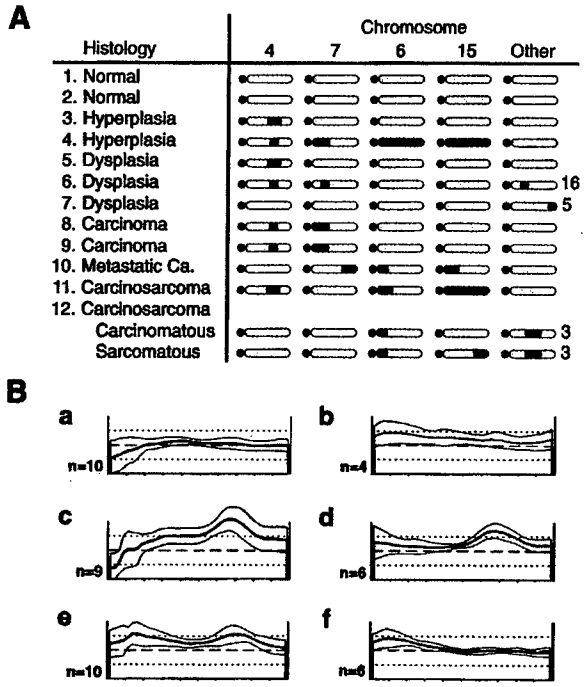

Figure 7. CGH Profiles

(A) Genomic changes seen in the mammary glands of 12 individual *Str1* transgenic mice. Samples 1, 3, 10, and 11 were from one transgenic founder line; samples 4 and 8 were from another line; and the remaining samples were from a third independent line. Approximate locations of macroscopic DNA gains (green) and losses (red) are indicated along otherwise unaltered (yellow) chromosomes, with black circles representing acrocentric centromeres. Sample 12, a carcinosarcoma, was microdissected and its carcinomatous and sarcomatous regions analyzed separately. All adjacent stromal and nonmammary control tissues had normal CGH profiles.

(B) Normalized fluorescence intensity profiles for chromosome 15 obtained with DNA isolated from (a) parental Scp2 cells, (b) a p2S7 cell–derived tumor, (c) preinduced p2S10 cells, (d) microdissected spindle-cell areas from a tumor derived from the same preinduced cells as in (c), (e) chondroid areas from the same tumor as in (d), and (f) normal stroma adjacent to the tumor in (d) and (e). Average green:red fluorescence ratios (heavy lines) ± 1 standard deviation (thin lines) are shown for the number of metaphase chromosomes examined (n). Dashed horizontal lines and upper and lower dotted lines indicate fluorescence ratios of 1, 1.5, and 0.5, respectively.

cancer syndromes result from gene defects that occur in stromal cells and induce stromal changes before epithelial abnormalities ever appear (Jacoby et al., 1997; Howe et al., 1998). These states of stromal remodeling and inflammation are precisely the conditions in which MMPs become upregulated (Mehindate et al., 1996). Thus, injury and inflammation may contribute to tumor development through MMPs that then promote the effects of carcinogens and preexisting gene defects. Likewise, the tumor promoter activity of phorbol esters may, in part, stem from their ability to upregulate stromal MMP expression (Gack et al., 1994).

Here we have shown that Str1 induces tumors in animals with and without an intact immune system. Using mammary epithelial cells that express *Str1* in a Tet-regulated manner, we found that Str1 converts functionally normal mammary epithelial cells into highly infiltrative mesenchymal-like tumors in vivo. In an independent set of experiments, we demonstrated that Str1 induces spontaneous neoplastic progression in the mammary glands of WAP-*Str1* transgenic mice. Although WAP-*Str1* and MMTV-*Str1* transgenic mice show similar proliferative changes in puberty (Sympson et al., 1994; Witty et al., 1995), neoplasms have not been described in MMTV-*Str1* mice. This may reflect the reported quenching of MMTV-driven transgene expression during pregnancy, strain differences, or the long latency and low incidence of tumor formation that one would expect to see based on our own results. In WAP-*Str1* mice, however, neoplastic changes arose without carcinogens

or preexisting mutations, were mild or absent in the absence of *Str1* expression, and were quenched by *TIMP1* expression. Thus, Str1 can promote mammary carcinogenesis by virtue of its proteolytic activity.

Several other observations support the participation of stromal MMPs early in cancer development. Wild-type fibroblasts foster the growth of human breast cancer cells in nude mice, yet fibroblasts lacking *MMP11/stromelysin-3* do not (Masson et al., 1998). *MMP1/collagenase-1* transgenic mice show increased sensitivity to chemical carcinogens (D'Armiento et al., 1995), while *MMP11* null mice have a reduced sensitivity to carcinogens (Masson et al., 1998). In addition, lack of either *MMP9/gelatinase B* or *Str1* slows the development of squamous carcinomas in human papilloma virus-16 transgenic mice (L. M. Coussens, D. Hanahan, and Z. W., unpublished observations). Epithelial MMPs may also contribute to tumorigenesis. Indeed, the lack of *MMP7/matrilysin* slows intestinal adenoma formation in mice carrying the Apc^{min} mutation (Wilson et al., 1997), and its overexpression within mammary glands accelerates mammary tumorigenesis in mice carrying an MMTV-*neu* transgene (Rudolph-Owen et al., 1998). Thus, several MMPs may contribute to early neoplastic progression.

EMT Is Related to Invasive and Migratory Behavior and MMP Expression

Our results also indicate that Str1 can trigger EMT in culture and in vivo. EMT occurs during normal embryonic development (Hay, 1995) and wound repair (SundarRaj et al., 1992) when adherent epithelia become migratory and invasive. Under such conditions, MMPs are highly expressed by adjacent mesenchymal cells (Chin and Werb, 1997). EMT also occurs in high-grade cancers (Birchmeier et al., 1996; Gilles and Thompson, 1996). The most aggressive human breast cancers undergo EMT, so they lack E-cadherin (Sommers et al., 1994), coexpress cytokeratins and vimentin (Domagala et al., 1990; Sommers et al., 1994), and express MMPs, such as *Str1*, that are otherwise confined to stromal cells (Ahmad et al., 1998; Martorana et al., 1998). Carcinosarcomas, which are among the most aggressive cancers, represent a compelling example of EMT (Gilles and Thompson, 1996). They are extremely uncommon in mice (Squartini and Pingitore, 1994) and account for only about 0.1% of all human breast cancers (Fisher et al., 1975), yet their rate of occurrence in *Str1* transgenic mice appears unusually high. As their name implies, carcinosarcomas are composed of distinct malignant cell populations that exhibit epithelial and mesenchymal features, respectively. Thus, it is not surprising that the mesenchymal-like cells of carcinosarcomas express MMP11 (Ahmad et al., 1998), which is normally confined to adjacent stromal cells. The acquisition of mesenchymal features is also consistent with the induced expression of MMP genes, including endogenous *Str1*, in *Str1*-transfected cells that have undergone EMT (Lochter et al., 1997). Altered E-cadherin and β-catenin expression are also hallmarks of EMT (Kim et al., 1998; Sun et al., 1998). Thus, the E-cadherin cleavage and β-catenin redistribution seen following *Str1* induction (Lochter et al., 1997) may have signaling implications for both EMT and tumorigenicity (Christofori and Semb, 1999).

How Do MMPs Foster Tumorigenicity?

MMPs are not mutagens. Therefore, they must promote tumor development by virtue of their ability to affect cellular signaling (Werb, 1997). MMPs can alter cell–cell and cell–ECM interactions and release bioactive fragments (Lukashev and Werb, 1998; Noe et al., 1999). For example, Str1 cleaves cell surface proteins, including E-cadherin (Lochter et al., 1997), a tumor suppressor (Christofori and Semb, 1999). MMPs also release growth factors, angiogenic factors, and their inhibitors from the ECM and cell surface (Patterson and Sang, 1997; Suzuki et al., 1997) and cleave growth factor binding proteins (Fowlkes et al., 1994) and receptors (Levi et al., 1996). They can induce a reactive stroma and cause recruitment of other host cells (Thomasset et al., 1998), and they generate cleavage products that may compromise cellular cytotoxicity (Kataoka et al., 1999). Thus, there are several ways in which MMPs can influence all stages of cancer progression, including initiation.

We favor the hypothesis that MMPs act to trigger the E-cadherin/β-catenin pathway, which can be linked to several aspects of cancer, including EMT, invasion, and genomic instability (Tlsty, 1998; Noe et al., 1999). In support of our hypothesis, the E-cadherin cleavage and β-catenin redistribution seen with Str1-induced EMT are accompanied by upregulation of *cyclin D1* (M. E. Lukashev and Z. W., unpublished results), a β-catenin-regulated oncogene (Tetsu and McCormick, 1999). Thus, Str1-induced E-cadherin cleavage may trigger both normal developmental and abnormal neoplastic changes.

MMPs also exert comparable effects in development and cancer. It is noteworthy that Str1 stimulates ductal proliferation and branching during puberty (Sympson et al., 1994; Witty et al., 1995) but induces apoptosis in anchorage-dependent secretory epithelium during pregnancy (Alexander et al., 1996; Thomasset et al., 1998). These seemingly contradictory actions can be reconciled by noting that the normal developmental fates of the target cells differ. Mammary ducts contain stem cells that are triggered to divide during branching morphogenesis. They also persist throughout involution, whereas alveolar cells do not. These effects are also consistent with the process of neoplastic transformation. The normal function of Str1 in inducing ductal proliferation and invasion during puberty is precisely what Str1 does to transformed mammary cells. Furthermore, although the induction of apoptosis in alveolar cells may defy tumorigenesis, it could also provide pressure for the selection of anchorage-independent, apoptosis-resistant clones and thereby foster tumorigenicity. Taken together with the propensity of Str1 to foster an altered stromal microenvironment (Thomasset et al., 1998), Str1 has the hallmarks of a multifactorial tumor promoter.

Do MMPs Stimulate Genomic Instability?

The presence of spontaneous tumors in *Str1* transgenic mice indicates that Str1 promotes either the accumulation of mutations or the survival of mutant cells. By altering cellular adhesion, MMPs could conceivably alter cell cycle checkpoint controls and promote genomic instability (Tlsty, 1998). The presence of recurrent DNA losses in both premalignant and malignant mammary lesions in *Str1* transgenic mice and of consistent DNA

copy number gains in undifferentiated tumors suggests that these loci contain recessive- and dominant-acting genes that contribute to early and late cancer progression, respectively. They also support the hypothesis that MMPs can produce an abnormal stromal environment within which clones of epithelial cells containing selected mutations may accumulate. It is intriguing that the WAP-*Str1* tumors are histologically diverse and arise late, suggesting a stochastic evolution, yet they exhibit stereotyped genomic changes, suggesting a common tumorigenic pathway. Several tumors did, however, exhibit the unifying feature of EMT together with consistent DNA gains on chromosomes 6 and 15. Although c-*myc* is located on mouse chromosome 15 (Adolph et al., 1987), its expression failed to correlate with DNA changes (M. D. S. et al., unpublished results). Thus, other relevant genes may reside at these loci.

Most of the genomic changes in the WAP-*Str1* transgenic mice were distinct from those in other transgenic mammary tumor models. For example, *p53*-deficient *Wnt-1* transgenic mice exhibit recurrent genomic changes on several chromosomes, including 4, but not 6, 7, or 15 (Donehower et al., 1995). Likewise, 82% of mammary tumors in MMTV-*neu* transgenic mice exhibit loss of heterozygosity on chromosome 4 (Ritland et al., 1997), and chromosome 4 deletions were also particularly prevalent in our own study. Thus, our results further implicate the middistal region of chromosome 4 as a potential tumor suppressor locus. Mammary tumors in SV40 T-antigen transgenic mice show consistent DNA gains in the telomeric region of chomosome 6 (Liu et al., 1998), rather than at its centromeric end as we observed in high-grade tumors of *Str1* transgenic mice. Thus, our data also indicate a number of novel loci of potential importance.

Implications for Human Cancer

If MMPs function in human cancer as they do in mice, then MMP mutations, amplifications, or polymorphisms may be associated with tumor development. Few studies have addressed this possibility despite the frequent cloning of MMPs as tumor-specific genes. Interestingly, a polymorphism in the human *MMP1* gene promoter that creates a transcription-enhancing Ets site occurs more often in tumor cell lines than in the general population (Rutter et al., 1998). This suggests that enhanced *MMP1* transcription may contribute to cancer susceptibility and supports the enhanced skin carcinogenesis seen in *MMP1* transgenic mice (D'Armiento et al., 1995). Epigenetic inactivation of the *TIMP3* promoter, seen often in human cancers (Bachman et al., 1999), could have similar implications. A functional polymorphism has also been found in the *Str1* promoter (Ye et al., 1996); however, its role in cancer remains unexplored.

Our results may also have implications concerning the therapeutic use of MMP inhibitors. Inhibition of Str1 by overexpression of TIMP1 quenched its ability to promote neoplasia in transgenic mice, indicating that active Str1 is required and that neoplasia can be suppressed if its activity is inhibited early on. Thus, a compelling argument could be made for inhibiting Str1 during any stage of tumor progression. However, once the neoplastic process was triggered in *Str1*-transfected cells, tumors could still form without continued *Str1* expression. Thus, our results also indicate that once Str1 effects alter the phenotype and genotype of mammary cells, its activity is no longer required for tumorigenicity. Likewise, cells induced to express *Str1* for 6 days in culture continue to undergo progressive EMT despite addition of Tet and an MMP inhibitor (Lochter et al., 1997). Thus, the converted cells may perpetuate further EMT by an MMP-independent feedback mechanism. This "hit-and-run" action of Str1 is also in keeping with the capacity of MMPs to affect signaling. Thus, although MMPs are expressed throughout tumor progression, and although MMP inhibitors may defy invasion, other stages of progression may become resistant to anti-proteinase therapy targeting Str1. Whether this is true for other enzymes remains to be determined.

Our findings thus indicate that Str1 can promote early neoplastic changes, stereotyped genomic changes, and late phenotypic conversions associated with aggressive tumor behavior. They also support the hypothesis that an altered stromal environment can promote neoplastic transformation. Elucidation of the pathways downstream from Str1 will be critical for defining new molecular targets.

Experimental Procedures

Cell Culture

Recombinant human Str1 (0.8 mg/ml; a gift from Dr. M. Navre, Affymax Research Institute) was prepared as described previously (Lochter et al., 1999) and activated by treatment with trypsin for 30 min at 37°C, followed by addition of soybean trypsin inhibitor. Scp2 cells were treated every other day for 6 days with 1 μg/ml activated Str1 in serum-free DMEM/F12 medium containing 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, and 50 μg/ml gentamicin with or without the hydroxamic acid metalloproteinase inhibitor GM6001 (10 μM; a gift from Dr. R. Galardy, Glycomed Corp.). All other culture and immunocytochemistry methods were performed as previously described (Lochter et al., 1997).

Tumorigenicity Assay

The developing epithelial parenchyma of abdominal (#4) mammary glands was removed from weanling *scid/scid* mice (DeOme et al., 1959), and 1×10^6 Scp2 or p2S cells in serum-free medium were injected into residual gland-free mammary fat pads or subcutaneously at the nape of the neck. Mice were maintained for 6 or more weeks with or without 10 μg/ml Tet in their drinking water. Inhibition of enzymatic activity by intraperitoneal injection of GM6001 (100 mg/kg/day) was not pursued, due to inhibited wound repair and postsurgical morbidity that was not seen for the carrier (4% carboxymethylcellulose in PBS). Tumor volumes were calculated as length × width^2/2.

Transgenic Mice

CD1 mice with an autoactivating rat *Str1* transgene targeted to mammary epithelium by the murine WAP gene promoter were generated as described (Sympson et al., 1994). Five independent transgenic founder lines (M2-5, M2-20, M2-21, M2-25, and M1-9), their nontransgenic littermates, and two transgenic sublines that had lost expression of the transgene (M2-5N and M2-21N) were analyzed. All mice were housed under similar conditions, and a similar fraction from each group (approximately one-third) was carried through pregnancy and lactation. The CA10 WAP gene promoter (Sympson et al., 1994) was also used to generate transgenic mice overexpressing a human *TIMP1* transgene (Alexander et al., 1996). These mice expressed human TIMP1 protein primarily during pregnancy and lactation (data not shown). WAP-*TIMP1* mice were crossed with one line (M2-5) of WAP-*Str1* mice to generate double transgenics. Half of these were carried through at least one pregnancy and lactation.

Histopathology

Mammary whole mounts (Sympson et al., 1994) were photodocumented and reprocessed for paraffin embedment. Hematoxylin/eosin, Masson's trichrome, and Alcian blue staining were by standard methods. Antigen retrieval was by brief 0.4 μg/ml proteinase K digestion for vimentin or by microwave heating in citrate buffer. Before adding peroxidase (HRP)-conjugated reagents, endogenous peroxidase activity was blocked with a methanol/H_2O_2 solution. Immunolocalization was by rat anti-mouse cytokeratin-8 (a gift from Dr. R. Kemler; 1:50) and biotinylated rabbit anti-rat IgG (Vector Laboratories; 1:200), HRP-conjugated mouse anti-bovine vimentin (DAKO; prediluted), or biotinylated rat anti-mouse smooth muscle actin (a gift from Dr. L. R. Lund; 1:50). Biotinylated antibodies were detected with avidin-biotin-HRP complexes. HRP activity was visualized with diaminobenzidine, and nuclei were counterstained with Meyer's hematoxylin.

Comparative Genomic Hybridization

DNAs were extracted from cultured cells, frozen tissues, or paraffin blocks by standard methods or from lightly stained paraffin sections after laser microdissection (Emmert-Buck et al., 1996). Reference and test DNAs labeled with Texas red-5-dCTP and fluorescein-12-dCTP, respectively, were hybridized to normal metaphase chromosome spreads; chromosomes were identified by 4,6-diamino-2-phenylindole (DAPI) counterstaining; and green:red fluorescence intensity profiles were obtained as previously described (Bain et al., 1997).

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The matrix metalloproteinase stromelysin-1 acts as a natural mammary tumor promoter

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Abstract

Extracellular matrix-degrading matrix metalloproteinases (MMPs) are invariably upregulated in epithelial cancers and are key agonists in angiogenesis, invasion and metastasis. Yet most MMPs are secreted not by the cancer cells themselves, but by stromal cells within and around the tumor mass. Because the stromal environment can influence tumor formation, and because MMPs can alter this environment, MMPs may also contribute to the initial stages of cancer development. Several recent studies in MMP-overexpressing and MMP-deficient mice support this possibility, but have required carcinogens or pre-existing oncogenic mutations to initiate tumorigenesis. Here we review the *spontaneous* development of premalignant and malignant lesions in the mammary glands of transgenic mice that express an autoactivating form of MMP-3/stromelysin-1 under the control of the whey acidic protein gene promoter. These changes were absent in nontransgenic littermates and were quenched by co-expression of a human tissue inhibitor of metalloproteinases-1 (TIMP-1) transgene. Thus by altering the cellular microenvironment, stromelysin-1 can act as a natural tumor promoter and enhance cancer susceptibility.

Matrix metalloproteinases (MMPs) are consistently upregulated in epithelial cancers, and considerable evidence indicates that they play an essential role in tumor angiogenesis, invasion and metastasis by virtue of their combined ability to degrade virtually all elements of the extracellular matrix (ECM) (Coussens and Werb, 1996). Indeed, without the help of ECM-degrading enzymes, cancer cells would probably be unable to cross the matrix barriers that otherwise contain their spread. This straightforward and conceptually appealing supposition forms the basis for current clinical trials of MMP inhibitors as anti-cancer agents. However, in addition to promoting cellular invasion by simply clearing away the surrounding matrix, MMPs can alter cellular signals (Lukashev and Werb, 1998; Werb, 1997) and may therefore influence initial tumor development. If so, then the inhibition of select MMPs during even the earliest stages of cancer progression may offer clinical benefit.

The MMP stromelysin-1 (MMP-3, Str1) exhibits a number of activities that would make it a particularly good tumor promoter. Like several other MMPs, *Str1* was first cloned and later recloned as a cancer-specific gene (Matrisian *et al.*, 1985; Muller *et al.*, 1988; Ostrowski *et al.*, 1988). In addition to degrading numerous ECM components, Str1 can activate gelatinase B and the collagenases, and can inactivate several serpin-type serine proteinase inhibitors (Sternlicht and Werb, 1999, for review). Moreover, it can release a number of cell surface molecules, including E-cadherin (Lochter *et al.*, 1997a), a known contributor to cancer development (Christofori and Semb, 1999; Tlsty, 1998).

Str1 is expressed by stromal cells during normal mammary gland development, and is strongly upregulated during post-lactational mammary involution when considerable ECM remodeling and alveolar apoptosis occur (Lund *et al.*, 1996; Thomasset *et al.*, 1998; Witty *et al.*, 1995). Interestingly, E-cadherin cleavage also occurs during involution and may induce apoptosis (Vallorosi *et al.*, 1999). Alternatively, ECM degradation may induce the apoptosis that occurs during involution. Either way, Str1 could act as an apoptotic stimulus. Indeed, Str1 does induce apoptosis in differentiated mammary alveolar epithelial

cells in culture and *in vivo*, however it also promotes the proliferation and branching of ductal epithelium (Alexander *et al.*, 1996; Boudreau *et al.*, 1995; Sympson *et al.*, 1994; Thomasset *et al.*, 1998; Witty *et al.*, 1995). These seemingly contradictory effects can be reconciled by noting that ductal epithelial cells normally divide during branching morphogenesis and persist throughout involution, whereas alveolar epithelial cells do not. Thus the differentiation status of the target cell may determine its response to Str1. These effects were first observed in transgenic mice with an autoactivating rat *Str1* transgene[1] targeted to mammary epithelium by the whey acidic protein (WAP) gene promoter (Sympson *et al.*, 1994) and mouse mammary tumor virus (MMTV) enhancer/promoter (Witty *et al.*, 1995). In these mice, *Str1* transgene expression resulted in increased ductal branching and precocious lobulo-alveolar development during puberty, basement membrane disruption and unscheduled involution during pregnancy, and alveolar collapse and low milk-protein production during lactation. Expression of the *Str1* transgene during pregnancy and lactation also led to enhanced expression of endogenous Str1 by mammary fibroblasts, collagen accumulation (fibrosis), neovascularization, and tenascin-C expression (Thomasset *et al.*, 1998). These changes are not only hallmarks of the reactive stroma seen during involution, but are also seen during cancer progression (Borsi *et al.*, 1992; Rønnov-Jessen *et al.*, 1996) and may even predispose toward neoplastic epithelial transformation (Jacobs *et al.*, 1999; Jacoby *et al.*, 1997; Kinzler and Vogelstein, 1998; Willenbucher *et al.*, 1999). Furthermore, the proliferative effects of Str1 could support neoplastic transformation and its apoptotic effects could help select apoptosis-resistant clones. Thus Str1 triggers a number of changes (increased cell proliferation, apoptosis, angiogenesis, and an altered stromal environment) that could potentially promote mammary carcinogenesis.

The above effects, which might be viewed as a prelude to cancer, were observed in transgenic animals under 4 months of age. To further address the potential tumor

[1] The autoactivating rat Str1 cDNA contained a Val^{92}-to-Gly^{92} transition within its propeptide domain, thus destabilizing the 'cysteine switch' that otherwise maintains enzyme latency (Sanchez-Lopez *et al.*, 1988).

promoting activity of Str1, mammary gland changes were monitored in WAP-*Str1* transgenic mice from 6-24 months of age. We observed the development of spontaneous premalignant lesions and mammary cancers in these mice and the virtual absence of such changes in their nontransgenic littermates and in related bitransgenic mice that co-express a human tissue inhibitor of metalloproteinases (TIMP-1) transgene under the control of the same promoter (Sternlicht *et al.*, 1999). These Str1-induced changes, which occur in the absence of exogenous mutagens or endogenous oncogene or suppressor gene defects, offer strong evidence that Str1 can indeed act as a natural tumor promoter.

Str1 Promotes Mammary Carcinogenesis

To evaluate the effects of prolonged Str1 expression in the mammary gland, WAP-*Str1* transgenic mice from five independent CD-1 founder lines and nontransgenic controls were maintained under similar conditions for up to two years (Sternlicht *et al.*, 1999). Only 12% of all WAP-*Str1* transgenic mice had histologically normal mammary glands. Instead, about three-quarters had moderate-to-severe fibrosis, about half had epithelial hyperplasias, 20% had atypical hyperplasias (dysplasias) or ductal carcinoma *in situ*, and 7% developed malignant mammary carcinomas (Table 1). Lymphocytic infiltrates accompanied these lesions in about half of all transgenic mice. By comparison, 87% of the nontransgenic mice had entirely normal mammary glands, and the remaining 13% had only mild fibrosis, hyperplasia or lymphocytic infiltration, and none of the more severe lesions seen in the animals expressing the Str1 transgene. These genotype-specific differences were highly significant ($p<0.002$ for carcinoma development and $p<0.0001$ for all other pathologies).

Approximately one-third of the mice from each group were carried through pregnancy and lactation. Parity had no effect on the already low incidence of mammary changes seen in the nontransgenic mice, and slightly increased the incidence of each type of lesion in the transgenic mice (Table 1). The hyperplastic and fibrotic lesions also tended to

be somewhat more severe in the parous subset of transgenic mice. The absence of more profound differences between parous and nulliparous mice, despite the use of a pregnancy-responsive promoter, probably reflects the low-level activity of the promoter during each estrus cycle which, in turn, would limit the increase in overall lifetime exposure to Str1 that would be gained through parity.

Abnormalities of varying severity were usually seen in all of the mammary glands examined in an individual transgenic mouse, and multiple abnormalities were often seen within individual mammary glands (Figures 1,2). Fibrotic changes included periductal, intralobular and diffuse accumulations of interstitial collagen and fibroblasts (Figure 1). In addition, fibrosis was often seen adjacent to or admixed with multiloculated adipocytes (Figure 2), a feature that may reflect the dedifferentiation of adipocytes towards a matrix-producing fibroblastic phenotype. Hyperplastic lesions included discrete hyperplastic alveolar nodules (HANs), multifocal and diffuse alveolar hyperplasias, adenomatous hyperplasias, and papillary ductal hyperplasias (Figures 1-4). Alveolar-type hyperplasias were most common. These were packed with otherwise normal alveoli containing a single layer of luminal epithelial cells surrounded by a single layer of myoepithelial cells (Figure 3). Several alveolar hyperplasias displayed evidence of secretory activity with apical lipid vacuolization of the luminal cells, luminal eosinophilic concretions resembling residual (inspissated) milk, and enlarged (ectatic) ducts containing proteinaceous material and lipid droplets (Figures 2,3). Papillary lesions, on the other hand, contained multilayered mounds of cells within distended ducts (Figure 4). In addition, myoepithelial cells were not only present in their normal position between the luminal epithelial cells and basement membrane, but were also abnormally located *within* the ducts as a result of the inward growth and folding of the papillary projections (Figure 4C-E). Dysplastic lesions also showed multiple cell layering, but with attenuated myoepithelial cell staining in some areas.

Twelve mammary carcinomas developed in the transgenic mice with only two arising before one year of age and an average tumor latency of 18.7 months. Hyperplastic

or dysplastic lesions and a fibrotic (scirrhous or desmoplastic) stroma were consistently found adjacent to the malignant tumors (Figures 1,5). For the most part, however, the tumors were histologically and cytologically diverse. One large adenocarcinoma with adjacent papillary lesions contained unusual, internally located myoepithelial cell islands (Figure 5). Otherwise, myoepithelial cells were uniformly absent in the tumors. The mesenchymal intermediate filament marker vimentin was absent in the nine tumors that were well- or moderately well-differentiated, except, of course, in their surrounding and intervening stroma (Figure 5H). The three remaining undifferentiated tumors, however, each exhibited vimentin immunoreactivity in addition to epithelial cytokeratin staining. One of these tumors gave rise to multiple lung and kidney metastases and stained positive for both vimentin and cytokeratins (Figure 6A-C). It also gave rise to a tumor cell line (TCL-1) (Lochter *et al.*, 1997b) that continued to express both intermediate filament markers in culture (Figure 6D-F) and formed highly metastatic spindle-cell tumors that remained cytokeratin- and vimentin-positive in nude mice (Figure 6G-I). The other undifferentiated tumors were carcinosarcomas (carcinomas with sarcomatous metaplasia) that contained distinct epithelial-like (carcinomatous) and mesenchymal-like (sarcomatous) cell populations (Figure 6J-L). The fibroblast-like sarcomatous cells had malignant cytologic features, composed the majority of some parts of the tumor, and contained similar genomic changes to those seen in the carcinomatous cells (Sternlicht *et al.*, 1999), thus indicating that they did not merely represent a stromal response to the malignant epithelial-like cells. Furthermore, both cell populations persisted after serial transplantation to immunocompromised mice. Thus, even though carcinosarcomas are extremely rare in humans and in mice, one-sixth of the tumors in WAP-*Str1* mice were of this type, and one-quarter of all tumors exhibited some degree of epithelial-to-mesenchymal phenotypic conversion. This incidence is intriguing in light of recent data indicating that phenotypically normal mammary epithelial cells undergo epithelial-to-mesenchymal conversion in response to Str1 in culture and *in vivo* (Lochter *et al.*, 1997a; Sternlicht *et*

al., 1999). This phenomenon has been associated with more aggressive malignant behavior (Birchmeier *et al.*, 1996; Gilles and Thompson, 1996), and careful examination reveals that a large percentage of human tumors, and perhaps all poorly differentiated tumors, exhibit some degree of epithelial-to-mesenchymal conversion (Oft *et al.*, 1998). Because most MMPs are stromal (mesenchymal) cell products, cancer cells begin to secrete their own MMPs only when they undergo such an epithelial-to-mesenchymal phenotypic transition (Ahmad *et al.*, 1998; Martorana *et al.*, 1998; Wright *et al.*, 1994). Thus, Str1 may represent both a cause and a consequence of epithelial-to-mesenchymal conversion.

TIMP-1 Inhibits Mammary Neoplasia in *Str1* Transgenic Mice

If the proteolytic activity of Str1 is responsible for the development of premalignant and malignant neoplasms in *Str1* transgenic mice, then these changes should be quenched by overexpression of its natural inhibitor, TIMP-1. We previously showed that mating the *Str1* transgenic mice with mice that overexpress a human *TIMP1* transgene driven by the constitutive β-actin gene promoter abolishes the ECM degradation and unscheduled apoptosis otherwise seen in young pregnant *Str1* transgenic mice (Alexander *et al.*, 1996). To test the ability of TIMP-1 to counter the long-term effects of Str1 in the mammary gland, WAP-*Str1* transgenic mice were crossed with mice that expressed the human *TIMP1* transgene under the control of the same WAP promoter. Using mammary hyperplasia as a surrogate end-point, 73% of 10-16-month-old offspring carrying the *Str1* transgene alone, but only 19% of age-matched bitransgenic mice carrying both the *Str1* and *TIMP1* transgenes developed hyperplasia ($p<0.02$, two-tailed Fisher's exact test). The double-transgenic mice also had a significantly lower incidence of hyperplasia than the whole cohort of WAP-*Str1* mice included in table 1 ($p<0.0006$). The few hyperplasias that did develop in the bitransgenic mice were considerably milder than those observed in the single-transgenic littermates with WAP-*Str1* alone. Parity did not significantly alter the incidence or severity of mammary hyperplasia in the single or double transgenic mice.

Mammary hyperplasias were also uniformly absent in those littermates with only the *TIMP1* transgene and in nontransgenic littermates. Thus it is the enzymatic activity of Str1 that is required to promote mammary neoplasia.

Other MMPs can Promote Carcinogenesis

Several recent observations support a role for MMPs early in cancer development. For example, mice that express a human collagenase-1 (MMP-1) transgene in squamous epithelium develop hyperproliferative skin lesions, and although they fail to form tumors spontaneously, they are more sensitive to chemical carcinogens than their non-transgenic littermates (D'Armiento *et al.*, 1995). Conversely, mice that *lack* stromelysin-3 (MMP-11) form fewer and smaller DMBA-induced tumors than wild-type mice (Masson *et al.*, 1998). Moreover, wild-type fibroblasts foster the tumorigenicity of human MCF-7 breast cancer cells in nude mice, whereas fibroblasts without MMP-11 do not (Masson *et al.*, 1998). Because ECM-associated growth factors are also required for MCF-7 tumorigenicity, the authors propose that MMP-11 may promote tumor formation by causing the release or activation of sequestered growth factors. The lack of matrilysin (MMP-7) in mice carrying the Apc^{min} mutation hinders the development of benign intestinal adenomas (Wilson *et al.*, 1997), and its overexpression in mammary tissue accelerates mammary tumor formation in mice carrying an MMTV promoter-driven *ErbB-2/neu* transgene (Rudolph-Owen *et al.*, 1998). In addition, MMTV-MMP-7 transgenic mice develop premalignant hyperplastic alveolar nodules (HANs) even in the absence of MMTV-*neu*, whereas their non-transgenic littermates do not (Rudolph-Owen *et al.*, 1998). The lack of either Str1 or gelatinase B (MMP-9) inhibits the development of human papilloma virus-16-induced squamous cell carcinomas in transgenic mice (LM Coussens, D Hanahan and Z Werb, unpublished observations). Furthermore, those tumors that develop despite the lack of MMP-9 tend to be more aggressive than usual, suggesting that MMP-9-deficiency provides pressure for the selection of less differentiated cancers that are better able to overcome the absence of

MMP-9 (unpublished observations). Other MMPs that are highly expressed in malignant disease, such as MMP-19 (Grant *et al.*, 1999)[2], may also influence cancer progression, but remain essentially unexplored.

Although the above studies support a role for MMPs in early tumor progression and indicate that MMPs may increase neoplastic risk, they still required pre-existing oncogene or suppressor gene mutations or the administration of chemical carcinogens to achieve tumorigenesis. Here, however, we have described the spontaneous development of Str1 transgene-induced lesions in the absence of such mutations or mutagens. These changes, which failed to occur in non-transgenic controls, were also quenched by coexpression of a TIMP-1 transgene. Thus their spontaneous development lends even greater support to the likelihood that MMPs profoundly influence early tumor initiation and development.

In addition to MMPs, closely related metalloproteinases, such as the membrane-anchored ADAM (a disintegrin and metalloproteinase domain) and the secreted ADAMTS (thrombospondin domain-containing) proteins, are likely to influence cancer progression (Vazquez *et al.*, 1999; Werb and Yan, 1998). For example, tumor necrosis factor-α converting enzyme (TACE, ADAM-17) can clearly influence cancer progression. Recent data also suggest that an unidentified metalloproteinase causes Fas ligand to be shed from cells, thus enabling them to avoid Fas-mediated apoptosis (Mitsiades *et al.*, 1999). In addition, a unique metalloproteinase that is inhibited by TIMP-1 but not TIMP-2 causes cleavage and shedding of the extracellular domain of the *ErbB2/neu* growth factor receptor (Codony-Servat *et al.*, 1999). Such shedding, which is often observed in breast cancer patients, may have oncogenic consequences and may limit the efficacy of anti-*ErbB2*-directed therapy. Other ADAM and ADAMTS domains may also influence cancer progression. For example, the cysteine-rich domain of meltrin-α (ADAM-12) can support tumor cell adhesion (Iba *et al.*, 1999). Some members of these multi-gene families may even play conflicting roles in cancer due to the presence of domains with distinct biologic

[2] Because the GenBank sequences submitted as human MMP-18 and 19 are identical but substantially different from the *Xenopus* MMP-18 sequence, they are designated as MMP-19.

activities. For example, the potent anti-angiogenic activity of some ADAMTS (metallospondin) family members (Vazquez *et al.*, 1999) may exert tumor suppressive effects, while other domains may promote tumor progression.

TIMPs may Promote *and* Suppress Carcinogenesis by Distinct Mechanisms

If, in fact, MMPs promote carcinogenesis, then their endogenous inhibitors, the TIMPs, should defy cancer development. However, whereas some studies do suggest that TIMPs suppress tumor development, others do not. In support of a tumor suppressive role, antisense depletion of TIMP-1 renders murine 3T3 cells tumorigenic *in vivo* (Khokha *et al.*, 1989). In addition, the transformation-promoting activity of the prototypic tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) is inhibited by TIMPs 1 and 2 in culture (Shoji *et al.*, 1997). Thus, the well-known ability of TPA to promote tumors *in vivo* may be partly due to its ability to upregulate MMP gene expression (Gack *et al.*, 1994; Reichardt *et al.*, 1998). On the other hand, TPA also upregulates TIMP-1 gene expression (Logan *et al.*, 1996; Lu *et al.*, 1991). In double transgenic mice, TIMP-1 overexpression inhibits simian virus 40 T antigen-induced hepatocellular carcinogenesis by inhibiting hepatocyte proliferation and angiogenesis (Martin *et al.*, 1996; Martin *et al.*, 1999). TIMP-1 overexpression also inhibits the tumorigenicity of melanoma and lymphoma cells (Khokha, 1994; Krüger *et al.*, 1997). However, in an experimental metastasis assay, certain tumor cell lines were better able to grow in the presence of tumor-associated TIMP-1, suggesting that it may protect ECM or cell surface molecules that are critical for cell viability (Soloway *et al.*, 1996). TIMP-1 overexpression also appears to *promote* intestinal adenoma formation in *Min* mice, yet a synthetic MMP inhibitor decreases tumor multiplicity in this same model (Heppner Goss *et al.*, 1998). This discrepancy may reflect the growth-promoting activity of TIMP-1, a function that point-mutation studies indicate is independent of its MMP-inhibitory activity (Chesler *et al.*, 1995). Indeed, TIMP-1 was initially cloned as "EPA" by virtue of its erythroid-potentiating activity (Docherty *et al.*, 1985) and has

been shown to act as a mitogen for other cell types (Bertaux *et al.*, 1991). Thus it is not entirely counterintuitive that TIMP-1 is often *upregulated* in human cancers (Kossakowska *et al.*, 1996; Lindsay *et al.*, 1997; Yoshiji *et al.*, 1996) and that such upregulation is predictive for metastatic progression and a poor prognosis (Jung *et al.*, 1997; McCarthy *et al.*, 1999; Mimori *et al.*, 1997; Ree *et al.*, 1997; Zeng *et al.*, 1995). Although TIMP-1 upregulation may simply be a consequence of the increased matrix remodeling that occurs during invasion, and would certainly hinder the pro-oncogenic and pro-invasive effects of MMPs, emerging evidence indicates that TIMP upregulation could also benefit tumors. In addition to its growth-stimulatory activity, recent studies indicate that TIMP-1 can upregulate vascular endothelial growth factor expression (Yoshiji *et al.*, 1998), that it can exert anti-apoptotic activity (Guedez *et al.*, 1998a; Guedez *et al.*, 1998b), and that it may even be internalized by cells and translocated to the nucleus (Ritter *et al.*, 1999).

Like TIMP-1, TIMP-2 promotes cell growth in culture (Hayakawa *et al.*, 1994; Nemeth *et al.*, 1996; Stetler-Stevenson *et al.*, 1992) and appears to inhibit tumor growth *in vivo* (Imren *et al.*, 1996). Although some studies indicate that TIMP-2 expression tends to be similar in tumors and matched normal tissues (Stetler-Stevenson *et al.*, 1990), others have found a significant correlation between TIMP-2 expression and the development of distant metastases (Ree *et al.*, 1997). Unlike TIMP-1, TIMP-2 expression is downregulated by TGF-β1 and is unaffected by serum and phorbol esters, each of which increase TIMP-1 expression (Leco *et al.*, 1992).

The role of TIMP-3 in cancer is also unclear. Some studies indicate that TIMP-3 is upregulated in human tumors (Uría *et al.*, 1994) and may provide an early marker for malignant disease (SP Hawkes, personal communication). Others, however, indicate that the TIMP-3 gene promoter is epigenetically *downregulated* during cancer development (Bachman *et al.*, 1999). Like TIMP-1, TIMP-3 is induced during cell transformation in culture (Lu *et al.*, 1991; Staskus *et al.*, 1991). TIMP-3 is also transiently induced by hepatocyte growth factor (HGF) (Castagnino *et al.*, 1998), which, in turn, has been

implicated in mesenchymal-to-epithelial cellular conversion (Tsarfaty *et al.*, 1994). Interestingly, ectopic overexpression of TIMP-3 can also induce mesenchymal-to-epithelial conversion and loss of malignant characteristics in cultured sarcoma cells, and its antisense depletion has the opposite effect, suggesting that it may be a mediator of HGF activity (Castagnino *et al.*, 1998). In addition, TIMP-3 is the only known endogenous inhibitor of TACE (Amour *et al.*, 1998). Thus, it may also influence cancer development by inhibiting TACE and other relevant ADAM and ADAMTS family members. Alternatively, some ADAM and ADAMTS proteins may be inhibited by other TIMPs. For example, aggrecanase-1 (ADAMTS-4) is inhibited by TIMP-1 (Tortorella *et al.*, 1999). Finally, the most recently discovered TIMP, TIMP-4, has been shown to inhibit mammary tumor growth and may be downregulated in human breast cancers (Wang *et al.*, 1997). Thus it appears that the issue of whether TIMPs defy or exacerbate the effects of cancer-causing agents and mutations is confounded by their multiple and independent functions. Ultimately, the TIMPs may both defy carcinogenicity through their metalloproteinase-inhibitory activity and promote it through their capacity to affect cellular behavior in a metalloproteinase-independent manner.

How MMPs Might Promote Tumor Development

Although MMPs are not oncogenic or mutagenic *per se*, there are several routes whereby they can alter cell signaling and thus affect the process of neoplastic transformation. By degrading extracellular matrices, MMPs alter cell-matrix interactions and cause the release of bioactive ECM fragments (Lukashev and Werb, 1998). MMPs can also cleave a growing list of cell surface molecules, including the tumor suppressor E-cadherin (Sternlicht and Werb, 1999). They can release active growth factors, angiogenic factors and angiogenic inhibitors from the cell surface and ECM (Patterson and Sang, 1997; Suzuki *et al.*, 1997). They can cleave growth factor binding proteins (Fowlkes *et al.*, 1994) and cell surface growth factor receptors (Levi *et al.*, 1996). They can generate

an α1-antitrypsin cleavage product that assists tumor growth and invasion, possibly by modulating NK cell cytotoxicity (Kataoka *et al.*, 1999). They can foster the recruitment of various host cells by altering the stromal environment (Thomasset *et al.*, 1998; Werb, 1997), and they may alter cell cycle checkpoint controls and promote genomic instability by affecting cell adhesion (Tlsty, 1998). MMPs can also induce programmed cell death in anchorage-dependent cells (Alexander *et al.*, 1996; Thomasset *et al.*, 1998), which could either defy tumor progression or exert pressure for the selection of anchorage-independent and apoptosis-resistant subpopulations, and thus promote progression. Therefore, MMPs may contribute in multiple ways to all stages of cancer progression, including initiation.

The evolution of epithelial cancers is also profoundly reliant on the stromal cells that help make up the tumor mass (Rønnov-Jessen *et al.*, 1996). In addition, an altered stromal environment may actually promote neoplastic transformation (Jacobs *et al.*, 1999; Jacoby *et al.*, 1997; Kinzler and Vogelstein, 1998; Willenbucher *et al.*, 1999). Indeed, stromal changes appeared to presage malignant epithelial changes in the WAP-*Str1* transgenic mice (Thomasset *et al.*, 1998). Thus, because Str1 can alter the extracellular environment and is itself a stromal product, it may be partly responsible for the tumorigenic effects of an altered stroma.

One of the more appealing prospective mechanisms that might be responsible for the tumor promoting capacity of Str1 is that it may alter E-cadherin/β-catenin signaling (Figure 7). According to this putative scenario, cleavage of E-cadherin by Str1 or another MMP may increase the cytosolic levels of its intracellular partner, β-catenin. Cytosolic β-catenin, in turn, can be phosphorylated and degraded, or translocated into the nucleus where it then partners with TCF/LEF transcription factors in order to regulate the transcription of genes that contain functional LEF recognition sequences within their promoters (Tlsty, 1998). In support of this mechanism, Str1-induced epithelial-to-mesenchymal conversion is accompanied by E-cadherin cleavage and a rapid redistribution of β-catenin away from cell-cell contacts towards a more cytoplasmic and perinuclear

location (Lochter *et al.*, 1997a). Furthermore, Str1 induces an early and sustained upregulation of *cyclin-D1* (ME Lukashev and Z Werb, unpublished results). This is consistent with the above mechanism, because *cyclin-D1* is regulated by β-catenin (Tetsu and McCormick, 1999) and can exert oncogenic effects in the mammary gland (Wang *et al.*, 1994). The c-*myc* proto-oncogene is also regulated by β-catenin/LEF transactivation in colon cancer cells (He *et al.*, 1998), however significant changes in c-*myc* expression were not observed by us during Str1-induced phenotypic conversion in mammary epithelial cells (unpublished results). Finally, matrilysin (MMP-7) gene expression is also regulated by β-catenin/LEF transactivation (Crawford *et al.*, 1999), and this same pathway may also account for our observation that a number of other MMPs are upregulated during Str1-induced epithelial-to-mesenchymal conversion (Lochter *et al.*, 1997a).

The ability of MMPs to release growth factors from the cell surface and ECM is also likely to play a critical role in cancer development. Some of these growth factors may influence tumor cells directly, while others may influence neighboring cells, such as endothelial cells, that are required to support tumor growth. Indeed, there is a growing awareness that MMPs promote tumor angiogenesis. In a transgenic model of pancreatic islet cell carcinogenesis, broad-range MMP inhibition suppresses the "angiogenic switch" that occurs during premalignant cancer progression and slows tumor growth during later stages of progression (Bergers *et al.*, 1999). Gelatinase B is probably an important target of such inhibition, in light of its association with premalignant angiogenesis (Coussens *et al.*, 1999) and its critical role in angiogenesis during bone development (Vu *et al.*, 1998). On the other hand, some MMPs, such as metalloelastase (MMP-12), matrilysin and gelatinase B, can cleave plasminogen to generate the angiogenesis inhibitor angiostatin (Patterson and Sang, 1997).

MMPs could also conceivably promote genomic instability by affecting adhesion-dependent cell cycle checkpoint controls (Tlsty, 1998). Interestingly, statistically nonrandom genomic changes were observed by comparative genomic hybridization in both

premalignant and malignant mammary gland lesions in WAP-*Str1* transgenic mice (Sternlicht *et al.*, 1999). The most prevalent change was a deletion in the mid-distal region of mouse chromosome 4 that was present in 70% of the examined WAP-*Str1* mammary lesions. This is consistent with the high incidence of chromosome 4 losses seen in two other models of mouse mammary cancer (Donehower *et al.*, 1995; Ritland *et al.*, 1997). In addition, a more recent study indicates that, in one of these models, the highest incidence of loss of heterozygosity occurs in the same mid-distal region of chromosome 4, thus further implicating this region as a putative tumor suppressor locus (Cool and Jolicoeur, 1999). Our own data are also consistent with the possibility that Str1 promotes the accumulation of genetic mutations or the selection and clonal expansion of mutant cells.

MMPs clearly do more than just degrade extracellular matrices, and such matrices are not just passive structures. MMPs can influence cell-matrix, cell-cell and paracrine signals that, in turn, control such basic processes as cellular growth, differentiation, morphogenesis, migration and death. Thus, the importance of MMPs in normal physiologic processes and in pathologic processes other than cancer may also partly stem from their ability to alter cellular signals. Moreover, the role of MMPs in normal physiologic processes and the potential for untoward effects must be considered when designing and undertaking clinical interventions that target the MMPs. A better understanding of the molecular mechanisms responsible for their expanding role in cancer can only benefit the development of more effective therapeutics and therapeutic stratagies.

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Table 1. Incidence of mammary gland pathologies in WAP-*Str1* transgenic mice.

	WAP-*Str1* Transgenic Mice			Nontransgenics	
	Virgin	Parous	All Mice	All Mice	P*
N	105	58	163	119	
Median Age (mos.)	18	17	18	18	
No Pathology	17 (16%)	3 (5.2%)	20 (12%)	104 (87%)	<0.0001
Fibrosis	74 (70%)	51 (88%)	125 (77%)	8 (6.7%)	<0.0001
Lymphoid Infiltrates	51 (49%)	36 (62%)	87 (53%)	10 (9.3%)	<0.0001
Hyperplasia	65 (62%)	40 (69%)	105 (64%)	6 (5%)	<0.0001
Dysplasia	18 (17%)	15 (26%)	33 (20%)	0 (0%)	<0.0001
Carcinoma	6 (5.7%)	6 (10%)	12 (7.4%)	0 (0%)	0.0016

* Two-tailed Fisher's exact test versus all transgene-expressing mice.

Figure Legends

Figure 1 Masson's trichrome-stained mammary gland sections from (**A**) nontransgenic and (**B-D**) WAP-*Str1* transgenic mice. The normal nontransgenic gland contains relatively few resting ducts surrounded by scant periductal collagen and embedded in an adipose stroma, whereas each transgenic gland exhibits extensive accumulation of blue-stained collagen (fibrosis) and few residual adipocytes. (**B**) This gland from a 7-month-old transgenic mouse contains numerous collapsed alveolar structures and extensive periglandular fibrosis. (**C**) A large dilated duct containing proteinaceous secretory material and hyperplastic alveolar epithelial cells with secretory vacuolization are apparent in this gland even though this 16-month-old transgenic mouse had never been pregnant. (**D**) This section from a 10-month-old transgenic mouse contains secretory hyperplastic epithelial cells and fibrosis adjacent to a secretory adenocarcinoma. Scale bar, 150 μm.

Figure 2 Carmine-stained wholemount (**A,B**) and H&E-stained paraffin section (**C-E**) of an abdominal (#4) mammary gland with diffuse hyperplasia (hp), fibrosis (fi) and lymphocytic infiltration (ly) from a 15-month-old parous WAP-*Str1* mouse sacrificed 4 months after its pups were removed. The hyperplastic branches indicated by the arrow in **A** are outlined in **C** and are shown at higher magnification in panels **B** and **D**. These sparse and disproportionately short secondary branches terminate in relatively well-developed lobuloalveolar structures and are surrounded by multilocular adipocytes (asterisk). The boxed area to the left of the central lymph node (LN) in **C** is enlarged in **E** and shows three hyperplastic areas, each with a distinct histologic appearance. Dilated (ectatic) primary ducts (du) containing considerable amounts of residual secretory material are also evident throughout the gland. Scale bars, 5 mm (**A,C**), 500 μm (**B,D,E**).

Figure 3 Histologic appearance of hyperplastic alveolar nodules (HANs) from 23- (**A**), 16- (**B**), 24- (**C**) and 12-month-old (**D-F**) virgin WAP-*Str1* transgenic mice as seen by wholemount (**A-C**), H&E (**D**), anti-cytokeratin-8 immunoperoxidase (**E**) and anti-smooth muscle actin immunoperoxidase (**F**) staining. The multiple alveolar structures are composed of an internal layer of cytokeratin-8-positive luminal epithelial cells with lipid vacuolization (**E**) surrounded by a single smooth mucle actin-positive myoepithelial cell layer (**F**). Adjacent normal areas (nl) contain mostly adipocytes and sparse ducts with the same bilayered luminal and myoepithelial cell staining, but without secretory vacuoles. LN, lymph node. Scale bars, 2 mm (**A-C**), 100 μm (**D-F**).

Figure 4 Histologic appearance of a florid papillary hyperplasia (intraductal papillomatosis) in the mammary gland of a two-year-old virgin WAP-*Str1* transgenic mouse as seen by wholemount (**A**), H&E (**B**,**C**), anti-smooth muscle actin (**D**) and anti-cytokeratin-8 (**E**) staining. The area outlined in **B** is shown at higher magnification in panels **C-E**. The small, basophilic cells within the papillary projections (me) are smooth muscle actin-positive (**D**) and cytokeratin-8-negative (**E**), indicating the abnormal, internal presence of myoepithelial cells. Although small focal collections of lymphocytes were present (not shown), fibrosis was not observed and the far ends of the gland were essentially normal. Scale bars, 2 mm (**A**,**B**), 200 μm (**C-E**).

Figure 5 Histologic appearance of moderately well-differentiated mammary adenocarcinomas from 15- (**A**,**B**), 19- (**C-E**) and 23-month-old (**F-H**) WAP-*Str1* transgenic mice as seen after wholemount (**A**), H&E (**B**,**C**,**F**), anti-cytokeratin-8 (**D**,**G**), anti-smooth muscle actin (**E**) and anti-vimentin (**H**) staining. (**A**,**B**) The tumor at right contains cystic spaces and necrotic debris, and sits adjacent to a diffuse lactational-like hyperplasia (asterisk) and a lymph node (LN) that is surrounded by muscle. The strip of connective tissue and skeletal muscle (arrow) is indicated for orientation. (**C-E**) This complex tumor (at right in each panel) contains numerous cystic spaces and tumor cell nests composed of mixed small and large cell populations. The small cells are smooth muscle actin-positive (**E**) and have small nuclei with a dense chromatin structure. The larger cells are cytokeratin-8 positive (**D**), have larger nuclei with a more open chromatin structure, and exhibit distinct intercellular bridges. The tumor is surrounded by a fibrotic stroma, atypical papillary lesions (**C**) and areas of secretory hyperplasia (asterisk). (**F-H**) These serial sections show a papillary adenocarcinoma with large cytokeratin-positive tumor cells, numerous mitotic figures, and an abundant vimentin-positive stroma (st). Scale bars, 2 mm (**A**,**B**), 300 μm (**C**), 200 μm (**D**,**E**), 50 μm (**F-H**).

Figure 6 Histologic appearance of poorly-differentiated mammary tumors and a tumor-derived cell line from WAP-*Str1* transgenic mice. (**A**) This H&E-stained area of primary mammary cancer from a 16-month-old virgin mouse contains spindle-shaped cells (left) and polygonal, epithelial-like tumor cells at lower right. (**B**,**C**) These two lung metastases from the tumor in **A** also contain spindled and polygonal cancer cells and stain positive for both cytokeratins (**C**) and vimentin (not shown). (**D-F**) The tumor cell line (TCL-1) established from the tumor in **A** exhibits a spindle-cell morphology and cytokeratin (**D**) and vimentin (**E**) immunoreactivity by dual immunocytochemistry and DAPI counterstaining (**F**). (**G-I**) TCL-1-derived tumors in immunocompromised mice show a pure spindle-cell morphology and numerous mitotic figures by H&E (**G**) and continue to stain positive for both cytokeratins (**H**) and vimentin (**I**). (**J-L**) Serial sections of this carcinosarcoma from a 17-month-old virgin transgenic mouse reveal a mixed cellular morphology by H&E (**J**) with polygonal carcinomatous cells that are cytokeratin-positive (**K**) and spindle-shaped sarcomatous cells that are vimentin-positive (**L**). Scale bars 100 μm (**A**,**C**), 200 μm (**B**), 50 μm (**D-I**), 75 μm (**J-L**).

Figure 7 A hypothetical model of how Str1 may affect cellular behavior via the β-catenin/LEF signal transduction pathway. Following E-cadherin cleavage by Str1 or another metalloproteinase, free cytosoic β-catenin pools are increased. In the absence of Wnt signaling, glycogen synthase kinase 3β (GSK-3β) phosphorylates β-catenin, thus targeting it for association with the adenomatous polyposis coli (APC) gene product and axin, ubiquitination, and proteosomal degradation. Alternatively, unphosphorylated β-catenin enters the nucleus where it interacts with LEF/TCF transcription factors, thus regulating the transcription of genes containing functional LEF recognition sites. A number of potential target genes are shown, although only *c-myc*, cyclin-D1 and matrilysin (MMP-7) have been so far shown to respond to β-catenin/LEF transactivation. Molecules indicated in red have been shown to play a causal role in cancer development. Frz, Frizzled family Wnt/Wg receptor; Dsh, Disheveled family or other GSK inhibitor; Wg, Wingless; ms, mouse.